THE EMBO JOURNAL

Manuscript EMBO-2011-79888

A transcriptional repressor co-regulatory network governing androgen response in prostate cancers

Kern Rei Chng, Cheng Wei Chang, Si Kee Tan, Chong Yang, Shu Zhen Hong, Noel Yan Wei Sng, and Edwin Cheung

Corresponding author: Edwin Cheung, Genome Institute of Singapore

Review timeline:

Submission date:	20 October 2011
Editorial Decision:	17 November 2011
Author Correspondence:	29 November 2011
Editorial Correspondence:	01 December 2011
Revision received:	14 March 2012
Accepted:	29 March 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 17 November 2011

Thank you very much for submitting your research manuscript the role of HDACs and EZH2 in the control of AR-driven transcription for consideration to The EMBO Journal editorial office.
I did receive comments from three scientists that confirm potential interest in your findings. However, their reports also reveal significant further experimentation would be needed to substantiate the elucidated repressor co-regulatory network, particularly the interesting proposal of HDACs and EZH2 to indeed abrogate AR-transcriptional output. Both ref#1 and #2 thus demand minimally the corroboration in further representative cell lines, according to ref#3 in fact ideally with evidence from prostate tumors. Further efforts should aim at comparing/contrasting your findings with existing datasets. As the comments seem overall encouraging and constructive, we decided to offer you the chance to address these critical issues during major revisions. Please be reminded that definitive insight(s) and functional corroboration serious concerns, particularly from the perspective and scope of our relatively broad and very competitive title. I thus urge you to approach these thoroughly to avoid disappointments much later in the process.
I do understand that this appears very demanding and would involve considerable further experimentation. We would therefore also understand if you were to take the current dataset for more rapid publication elsewhere. In case you decide to revise for The EMBO Journal, I am happy to discuss/clarify specific points also with regard to the potential timeline in more detail. Please do not hesitate to contact me (preferably via E-mail).

Lastly, I do have to formerly remind you that it is EMBO_J policy to allow a single round of revisions only and that the ultimate decision depends on the content and strength of your adequately modified version.

I am very much looking forward to your response and remain with best regards.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this work, the authors have examined by using genome-wide approaches potential cross-talk between androgen receptor (AR) and ERG as well as several transcriptional co-repressors; HDAC1, HDAC2, HDAC3, and EZH2. Collectively, their results show that ERG, working together with co-repressors (HDACs and EZH2) are involved in the regulation of AR-dependent transcription programs and that this regulation, for example, through suppression of vinculin gene expression, may be involved in increased cancer cell invasiveness.

By and large, the work is carefully conducted and some of the results are of potential interest to a larger audience, not only to androgen receptor aficionados. There are, however, a number of serious shortcomings in this work that need to be addressed, in order to pinpoint the novel features of the present study and to validate the authors' main conclusions.

Major concerns

1. With regard to the cross-talk between AR and ERG pathways, similar results have already been published by Yu at al. (Cancer Cell 17, 443-454, 2010). Even though the authors cite this paper in a number of instances, they fail to compare their own results to those of Yu et al. In other words, the novel aspects or results dissimilar to those of Yu et al. need to be made clear in the present manuscript.

2. Only a single cell line (the VCaP) cells were used in the present study. Since the authors make on several occasions references to prostate cancer progression, it would be important to know whether or not the results are specific to VCaP cells only or also applicable to other prostate cancer cell lines with a lower level of ERG expression.

3. The data in Fig. 2 show very convincingly that there is colocalization between a subset of AR and ERG cistromes and that there are sites that are unique to each of the two cistromes. What is missing is the connection of the two classes of AR-binding sites to androgen-dependent transcription programs. In other words, are AR unique sites and AR+ERG sites linked to the same or different transcription programs, as determined by microarray analysis. The authors state in the Materials and Methods section that microarray analyses were performed.

4. The results from siERG experiments are shown for two loci only (KLK3 and FKBP5). Since there is a significant overlap between AR and ERG cistromes in VCaP cells, it is important to show a genome-wide view of AR-binding sites upon ERG knock-down. Were there both qualitative and quantitative changes? Likewise, a more comprehensive analysis of the changes in androgen-dependent gene expression upon ERG knock-down is needed. In the present study, the authors provide only a few examples for genes whose androgen-dependent up-regulation is attenuated by the presence of ERG. What is the situation with genes down-regulated by androgen? Is their expression or androgen responsiveness perturbed by ERG knock-down?

5. The authors state in the abstract that "these corepressors function as a multiprotein complex to enhance ERG-mediated repression of AR-induced transcription..." The presence of these multiprotein complexes is not shown by direct experiments in this study.

6. Even though the ChIP-seq data look very good, the authors should nevertheless present information on the specificity of the antibodies used, together with validation by direct ChIP assays

of some of the AR sites that overlap with ERG or corepressor sites.

7. Experiments on vinculin expression yielded interesting results. However, the conclusion from simple Matrigel studies that silencing of vinculin "leads to increased cancer cell invasiveness" appears to be an overstatement.

8. In the Discussion section (pg. 16), the authors deal with ERG binding at promoters of AR target genes. The results cited in this context need to be shown either in the main body of the manuscript or as supplementary information.

Minor concerns

1. In their discussion of the role of EZH2 in prostate cancer, the authors need to cite Min et al. (Nature Medicine 16, 286-294, 2010).

2. On pg. 5, line 3 from bottom: DHT-dependent up-regulation of ERG mRNA and protein accumulations peaked at 12 h and 24 h (not at 6 h and 12 h, as mentioned in the text).

3. Many of the figure legends should be more comprehensive. In addition, in a few instances, various subpanels of a figure are not dealt with in the text in the order that they are labeled.

4. In Fig. 3, panel C, it appears that silencing of AR has only marginal effect on the level of ERG protein without or with DHT exposure. It there a good explanation for this?

5. Panel A in Fig. 5 is totally unclear for an uninitiated reader. What are these sites and how are they represented in these figures?

6. The typescript contains a number of grammatical and/or typographical errors.

Referee #2:

A putative functional interaction between the androgen receptor (AR) and ERG has been previously proposed, but the underlying consequence and mechanisms of AR-ERG interplay remain poorly defined. Given the high frequency of ERG upregulation in human prostate cancers (including via AR-dependent TMPRSS2-ERG fusions), molecular dissection of the putative AR-ERG interaction is of potentially strong translational relevance. The present study utilizes a series of genome-wide analyses to probe the AR-ERG interaction in cells with the TMPRSS2-ERG fusion product. Key stated findings are that: distinct but overlapping chromatin binding events for AR and ERG are observed upon androgen (DHT) stimulation, ERG attenuates AR signaling by suppression of AR binding, histone deacetylases and the histone methytransferase EZH2 are recruited to loci with dual AR and ERG binding (thus assisting in attenuation of AR signaling), and ERG/HDAC/EZH2 cooperate to suppress AR-dependent exprssion of adhesion molecules (thus promoting invasive-like phenotypes).

Critique: This is a novel, well-written, and generally well executed study that would be of interest to the nuclear receptor and prostate cancer fields. The data shown are mostly supportive of the stated conclusions, and the findings are of molecular and translational impact. However, the following concerns should be addressed so as to further improve confidence in the findings:

1. Specificity is an issue. The major mechanistic studies herein rely on a single cell line (VCaP), and it is not clear whether the observed regulation of AR by ERG/HDAC/EZH2 is a fluke of this model or is commonly observed in ERG-high cells. The proposed pathway should be tested in a second model system. It is appreciated by this reviewer that VCaPs ar the major model representing fusion-positive disease; however, models of ERG upregulation in other prostate cancer cell backgrounds have been generated, and could be used or similar models made to assess the overall specificity of the proposed pathway.

2. Figure 4B- Kinetic analyses would be of benefit for this figure, so as to determine the point at which HDACs and EHH2 are recruited at sites of prostate cancer relevance (relative to AR and ERG).

3. Figure 7- These studies would benefit from additional consideration:
Panel b: Additional datasets should be shown and included here or in the supplement. Showing only one dataset from Oncomine raises concerns about the generality of the observation.
Panel C: The potential of a negative correlation between ERG and VCL is of interest but the data are not compelling as shown. First, additional datasets should be included. Second, validation of this anti-correlation by immunohistochemical analysis of tissue specimens would provide confidence in the stated conclusions.

Panel J: These data, which have the potential to provide biological relevance, are underdeveloped. Images should be shown to support the data shown, and alternative model systems should be used to assess the impact on cell migration/invasion.

Referee #3:

Critique:

This manuscript proposes an interesting and novel concept of how a repressor co-regulatory network governs androgen response in prostate cancers. Specifically, by using ChIP-Seq, extensive global binding maps were created of AR, ERG, and transcriptional corepressors in prostate cancer cells; HDAC1, HDAC2, HDAC3, and EZH2 were also assayed. They all seem to be directly involved in androgen-regulated transcription. It seems that ERG promotes prostate cancer progression by working together with HDACs and EZH2 in turn to directly attenuate the transcriptional output of AR.

Major points:

1. The notions summarized above go against the grain because of the generally accepted insight that INCREASED AR activity (such as AR amplification and activity as measured by PSA expression) is associated with prostate cancer progression, even including ablation resistant disease. These issues need to be explicitly and specifically addressed in terms of prostate tumors (and not only in cell lines).
2. The manuscript is littered with many imprecise and sloppy statements.

Minor points:

1. Paragraph 1 of the introduction relies on two old references (Heinlein & Chang, 2002 & 2004). Better (and more recent) references exist.
2. The color differences in Fig 1C are not distinct enough and can be confusing.
3. In Fig1 the expression analyses of the AR and ERG were at 6, 12 and 24 hours after DHT treatment, yet the times for the ChIP-seq analyses were done after 2 and 18 hours after DHT treatment. To interpret the latter the expression levels of the two transcription factors are needed at the same times, especially as expression changes quite significantly over time.
4. It is stated near the top of page 7: "Finally, we noticed that AR recruitment was significantly stronger at AR+ERG co-localized binding sites compared to AR unique sites (Fig. 2G)". What exactly is meant by "stronger"? Higher affinity or more sites occupied in the mixture of cells? Binding is typified by being "stronger" or "weaker" at many places throughout the manuscript.
5. Towards the bottom of page 7 it is stated: "...our AR ChIP-seq showed ARBS are preferentially located at distal regions that are far away from the transcriptional start sites (TSS) of genes (Fig.2E)". The % of binding sites at the different genomic regions needs to be compared with random regions of about the same size to be able to make the above statement (especially typifying the binding as "preferentially located").
6. Further down on page 7 it is stated: "In conservation analysis, AR and ERG are generally more conserved at the ChIP-Seq peak center relative to their flanking regions (Fig. 2F)." What else

was expected?
7. In the section "Transcriptional collaboration between AR and ERG", only two sites/genes were analyzed (PSA and FKBP5). More sites/genes need to be considered before the collaboration can be generalized.
8. Bottom of page 10 it is stated: "In contrast, ERG unique binding sites preferred to recruit HDAC1 and -2, but not EZH2." Personification of inanimate objects needs to be avoided and statements like these (also elsewhere) need to be rephrased.

Author Correspondence 29 November 2011

Thank you for the positive feedback on our manuscript and the opportunity to revise and resubmit our work.

We have carefully read your suggestions and the reviewers comments and have now begun to address the issues raised by the reviewers. Overall, we feel the request by reviewer #3 to prove our findings in prostate tumors, although important, seems quite unreasonable and beyond the scope of this manuscript. In the current manuscript, we have generated the most extensive genome-wide information on AR and corepressor proteins in cancer cells (a technical feat in itself). This information led us to reveal a novel mechanism of transcriptional regulation by AR in prostate cancer cells. Furthermore, we have included molecular and cellular evidence to support our genome-wide analysis. I believe the type of experiments the reviewer is requesting either requires prior collaborations with clinicians and/or animal studies and thus will take a very long time and the findings resulting from these experiments would be enough for another manuscript on its own. We feel our current contribution, although only in cell lines should be seen, tested and advanced by other researchers in the community. We hope you agree with this.

The request by reviewers #2 and #3 to repeat our experiments in another representative prostate cancer cell line which expresses AR, ERG, and the corepressors, is reasonable. However, we are currently having difficulty in addressing this point and we would like your input. While we would like to perform these experiments, we are currently unable because such cell lines are not readily available. From our literature search, we found the only cell line that is similar to the VCaP cells and has been used by other researchers is DUCaP. In fact, both the VCaP and DUCaP cell lines were established by the same investigator, Dr. Kenneth Pienta from University of Michigan. We wrote to Dr. Pienta recently and as you can see from our correspondence with Dr. Pienta below (in blue), he no longer provides the DUCaP cell line to the research community due to the difficulty in growing the cells. Moreover, he confirmed that the only available AR and ERG positive cell line is currently VCaP (which explains why most studies to date uses only VCaP cells). The scarcity of cell lines similar to VCaP is most likely because prostate cancer cell lines in general are notoriously difficult to generate and also the area of ERG biology in prostate cancer is still relatively new. We hope this limitation in reagent does not go against us and prevent our work from being published in EMBOJ.

Although, we cannot perform additional experiments in another VCaP-like cell line, we are currently performing additional experiments in the LNCaP cell line which is AR+ and ERG- (or expresses ERG at low levels). We think the results from the LNCaP experiments will provide addition insights and address the specificity issues raised by reviewers 1 and 2. In fact, reviewer 1 actually suggested performing experiments in an ERG-ve cell line as a potential alternative. Besides the LNCaP work, we will be doing additional experiments as requested by reviewers 1 and 2.

Please let us know what your thoughts are regarding the above two main points.

Thank you.

Editorial Correspondence 01 December 2011

Thank you very much for your letter outlining future experimentation for your revision. I fully accept the notion on the cell line issue and hope that the results form LNCaP cells would substantiate your very interesting proposal on AR-transcription.
Related to the situation in actual prostate tumors I am a bit more reluctant as this would significantly strengthen also the clinical relevance of your work. Please let me add that we would not aim at a full-fledged molecular analysis of patient samples, but corroboration of some of the findings (maybe exploring the vinculin aspect) in some tumor samples would be sufficient at this stage. This would be particularly interesting, given that your results go against the current dogma, thus multiple lines of evidence including some in fact (patho-) physiological support would extremely help the matter.

I hope that this clarified my initial decision.
Please do not hesitate to contact me in case of further questions.

Yours sincerely,

Editor

The EMBO Journal

1st Revision - Authors' Response 14 March 2012

We thank all the reviewers for their comments and suggestions. In this revised manuscript, we have included experiments as requested by the reviewers and provide additional data and explanations that address all their concerns.

Referee #1 (Remarks to the Author):

Major concerns:

1. *With regard to the cross-talk between AR and ERG pathways, similar results have already been published by Yu at al. (Cancer Cell 17, 443-454, 2010). Even though the authors cite this paper in a number of instances, they fail to compare their own results to those of Yu et al. In other words, the novel aspects or results dissimilar to those of Yu et al. need to be made clear in the present manuscript.*

We agree with the reviewer and have now included a detailed comparison of our AR and ERG findings with that of *Yu et al.* in the discussion section. Although the experimental design and ChIP-Seq conditions are quite different between the two studies (*i.e.* the antibodies used, the time the ChIPs were performed, *etc*), there was a significant overlap between the AR and ERG maps generated in both studies (Fig. S12). More importantly, both studies showed a large co-localization between the cistromes of AR and ERG. Beyond these similarities, our dataset provided several novel important findings over the work by *Yu et al*. In particular, because we performed our ChIP-Seq assay at different points after androgen stimulation, we were able to observe that ERG was in general already pre-bound to chromatin on a genome-wide level. Thus, our results suggest that ERG is a potential novel pioneer factor (akin to FoxA1) in AR-dependent transcription. This is an important and unexpected novel finding.

In addition to AR and ERG, our work provides the first and most extensive transcriptional co-repressor (HDAC1-3 and EZH2) cistromic maps in prostate cancer cells. Our results suggest the enhanced genome-wide recruitment of these frequently over-expressed transcriptional co-repressors

to AR+ERG binding sites after androgen stimulation as possible collaborating partners in the AR and ERG crosstalk.

2. *Only a single cell line (the VCaP) cells were used in the present study. Since the authors make on several occasions references to prostate cancer progression, it would be important to know whether or not the results are specific to VCaP cells only or also applicable to other prostate cancer cell lines with a lower level of ERG expression.*

This is a valid point. We have tried our best to find another suitable and readily available ERG-fusion positive prostate cancer cell line for the revision of our manuscript but unfortunately to no avail. After an extensive literature search, apart from VCaP, only DuCaP (Lee et al., In Vivo 2001) and NCI-H660 (Metz et al., Neoplasia 2007) over expresses TMPRSS2-ERG endogenously. NCI-H660 cells do not express AR and therefore is not suitable for assessing the crosstalk between AR and ERG. We requested Dr Pienta (who also established the VCaP cell line used in this study) for the DuCaP cell line but unfortunately, he has stopped providing this cell line to researchers due to technical difficulties in culturing these cells. Thus, we hope the reviewer will understand that due to reagent limitations we are currently not able to fully address this very important point.

With regards to using another cell line that expresses lower ERG levels, we have now repeated additional experiments using the LNCaP prostate cancer cell line, which over expresses the ETV1 but not the ERG fusion gene (Tomlins et al., Science 2005). Under the same ChIP condition, HDACs and EZH2 were recruited but generally weaker (based on % input) at ARBS that were previously examined in VCaP cells (Fig. S5). Similarly, when LNCaP cells were treated with HDAC or EZH2 inhibitors, there was either less effect or no significant increase on AR target gene expression (Fig. S6). Taken together, we believe these preliminary results suggest the possibility that the suppression of AR activity by HDACs and EZH2 might be more pronounced in an ERG-fusion positive prostate cancer environment, however, we acknowledge that further work will be needed to fully determine the differences between these two types of prostate cancers.

3. *The data in Fig. 2 show very convincingly that there is colocalization between a subset of AR and ERG cistromes and that there are sites that are unique to each of the two cistromes. What is missing is the connection of the two classes of AR-binding sites to androgen-dependent transcription programs. In other words, are AR unique sites and AR+ERG sites linked to the same or different transcription programs, as determined by microarray analysis. The authors state in the Materials and Methods section that microarray analyses were performed.*

We thank the reviewer for this suggestion. We have now associated androgen-regulated genes (>2 fold) from our microarray analysis with either AR+ERG co-localized or AR unique binding sites that are within 20 kb from TSS. We then performed gene ontology analysis on these two sets of genes using Ingenuity systems Pathway Analysis (IPA). Our results suggest that AR+ERG sites are more associated with transcription programs related to cellular movement, cellular growth and proliferation, cell cycle and cell morphology, while AR unique sites are more associated with a transcription program linked with cell death (Table S2).

4. *The results from siERG experiments are shown for two loci only (KLK3 and FKBP5). Since there is a significant overlap between AR and ERG cistromes in VCaP cells, it is important to show a genome-wide view of AR-binding sites upon ERG knock-down. Were there both qualitative and quantitative changes? Likewise, a more comprehensive analysis of the changes in androgen-dependent gene expression upon ERG knock-down is needed. In the present study, the authors provide only a few examples for genes whose androgen-dependent up-regulation is attenuated by the presence of ERG. What is the situation with genes down-regulated by androgen? Is their expression or androgen responsiveness perturbed by ERG knock-down?*

We agree. We have now expanded the binding analysis by ChIP-qPCR on an additional 18 ARBS after ERG knockdown. Consistent with our original observation, there was a general increase in AR binding after ERG knockdown at all the sites we tested (Fig. S2). Recently, a ChIP-Seq of AR after

ERG knockdown was published by *Yu et al* (Cancer Cell, 2010). We examined this dataset in detail and showed that the depletion of ERG increased the overall number of new AR binding events (Fig. S2). Taken together, our results in combination with *Yu et al* show there is both a qualitative and quantitative change in the AR cistrome after ERG knockdown.

To obtain a more comprehensive analysis of androgen-dependent gene expression upon ERG knock-down, we performed a microarray analysis of androgen stimulated VCaP cells before and after ERG depletion. Overall, our results showed that there were several distinct group of genes affected by ERG (Fig. S1). In addition to KLK3 and FKBP5 there was a large group of DHT upregulated genes (393) that was further induced upon ERG silencing. A smaller group of DHT upregulated genes (151) was repressed by ERG knockdown. As for DHT downregulated genes, 192 were further repressed while 86 were de-repressed after ERG knockdown.

5. *The authors state in the abstract that "these corepressors function as a multiprotein complex to enhance ERG-mediated repression of AR-induced transcription..." The presence of these multiprotein complexes is not shown by direct experiments in this study.*

We thank the reviewer for highlighting this mistake. We acknowledge our experiments do not provide direct proof that the corepressors function as a multiprotein complex. Accordingly, we have omitted the use of "mulitprotein complex" for describing the co-repressors.

6. *Even though the ChIP-seq data look very good, the authors should nevertheless present information on the specificity of the antibodies used, together with validation by direct ChIP assays of some of the AR sites that overlap with ERG or corepressor sites.*

We agree and have now included this information in the Material and methods and supplementary information sections. The AR antibody used in this study has been used in our previous genome-wide ChIP study (Tan et al., Mol Cell Biol. 2012) and is also widely used by many other researchers. For the ERG antibody, we showed that the maps generated in this study overlap substantially with the map generated by *Yu et al*., *Cancer Cell 2010* even though a different ERG antibody was used. For HDACs 1-3, the specificity of the antibodies used has been validated in *Wang et al., Cell 2009*. The anti-EZH2 we used has been used for ChIP assays in numerous studies (*i.e.* Lee et al., Mol Cell 2011 and van Dessel et al. Nucl. Acids Res. 2010). In our revised manuscript, we have also used another anti-EZH2 (39901, Active Motif) to validate our ChIP-Seq results (Fig. S4). *Finally, we independently validated the specificity of each antibody used for our Chip-Seq by silencing each factor in VCaP cells and performing a western blot analysis with the corresponding antibody (Fig. S14).*

As requested, we have performed direct ChIP assays on additional ARBS that overlap with ERG and the corepressors (Fig. S4).

7. *Experiments on vinculin expression yielded interesting results. However, the conclusion from simple Matrigel studies that silencing of vinculin "leads to increased cancer cell invasiveness" appears to be an overstatement.*

We agree and have now toned down our conclusion.

8. *In the Discussion section (pg. 16), the authors deal with ERG binding at promoters of AR target genes. The results cited in this context need to be shown either in the main body of the manuscript or as supplementary information.*

We have now included the percentage of androgen regulated genes with an ERG binding site at its promoters and compared it with all RefSeq genes. Our results showed that there is an enrichment of promoter ERG occupancy in androgen-regulated genes compared to all RefSeq genes (Fig. S13).

Minor concerns:

1. *In their discussion of the role of EZH2 in prostate cancer, the authors need to cite Min et al. (Nature Medicine 16, 286-294, 2010).*

We thank the reviewer for bringing this paper to our attention. This reference is now included in the introduction.

2. *On pg. 5, line 3 from bottom: DHT-dependent up-regulation of ERG mRNA and protein accumulations peaked at 12 h and 24 h (not at 6 h and 12 h, as mentioned in the text).*

We thank the reviewer for the spotting this mistake. We have made the changes accordingly.

3. *Many of the figure legends should be more comprehensive. In addition, in a few instances, various subpanels of a figure are not dealt with in the text in the order that they are labelled.*

We thank the reviewer for the suggestions. We have made the changes accordingly.

4. *In Fig. 3, panel C, it appears that silencing of AR has only marginal effect on the level of ERG protein without or with DHT exposure. It there a good explanation for this?*

We believe there is actually substantial (although incomplete) decrease in the level of ERG protein after AR silencing. The incomplete down regulation could be likely due to numerous potential reasons including incomplete AR knockdown, a long ERG protein half-life, or that other factors are also required for ERG expression which silencing AR alone will not completely down regulate ERG expression.

5. *Panel A in Fig. 5 is totally unclear for an uninitiated reader. What are these sites and how are they represented in these figures?*

We apologize for the lack of clarity to the legend of Fig. 5A and have now provided additional explanation. Essentially, HDAC1-3 and EZH2 ChIP-Seq peaks (with DHT stimulation) were analyzed using our bioinformatic tool, CentDist (Zhang et al., Nucl. Acids Res. 2011). Fig. 5A shows the average distribution of Androgen Response Elements (AREs) and ETS binding motif (ETS) centered at the peaks of the binding sites of the respective co-repressor. If the motif distribution peaked near the center of the binding sites of a particular co-repressor, then our results suggest that the co-repressor is recruited to a position near that motif.

6. *The typescript contains a number of grammatical and/or typographical errors.*

We have gone through the manuscript and made numerous corrections throughout.

Referee #2 (Remarks to the Author):

1. *Specificity is an issue. The major mechanistic studies herein rely on a single cell line (VCaP), and it is not clear whether the observed regulation of AR by ERG/HDAC/EZH2 is a fluke of this model or is commonly observed in ERG-high cells. The proposed pathway should be tested in a second model system. It is appreciated by this reviewer that VCaPs are the major model representing fusion-positive disease; however, models of ERG upregulation in other prostate cancer cell backgrounds have been generated, and could be used or similar models made to assess the overall specificity of the proposed pathway.*

We fully agree. Please see our response to Reviewer 1, comment #2.

2. *Figure 4B- Kinetic analyses would be of benefit for this figure, so as to determine the point at which HDACs and EZH2 are recruited at sites of prostate cancer relevance (relative to AR and ERG).*

We thank the reviewer for this suggestion. We have now performed ChIP of all the factors at 0, 15, 30, 60 and 120 min after DHT stimulation (Fig. S3). Our results showed that HDACs and EZH2 are recruited to AR and ERG co-occupied binding sites as early as 15 min after DHT stimulation, suggesting these factors are promptly recruited upon androgen signaling.

3. *Figure 7- These studies would benefit from additional consideration:*

Panel b: Additional datasets should be shown and included here or in the supplement. Showing only one dataset from Oncomine raises concerns about the generality of the observation.

We agree. We believe the reviewer may have originally missed this information. In addition to the study in the main figure, we had already included 3 other datasets from Oncomine in Fig. S2 (now Fig. S9). For the revised manuscript, we have now included the MSKCC prostate cancer dataset (Taylor et al., Cancer Cell 2010) as well. In summary, all of the datasets showed a similar trend, which suggests a general observation for vinculin in prostate cancer.

Panel C: The potential of a negative correlation between ERG and VCL is of interest but the data are not compelling as shown. First, additional datasets should be included. Second, validation of this anti-correlation by immunohistochemical analysis of tissue specimens would provide confidence in the stated conclusions.

As mentioned above, apart from the one study in the main figure, we had 3 (now 4) additional datasets in the supplementary section (Fig. S9). All of the datasets exhibited similar trends, suggesting the generality of our observation. With regards to performing immunohistochemical analysis on tissue specimens, we agree that this would further strengthen our claim. For the revision, we screened a panel of ERG and VCL antibodies but could not find a VCL antibody that performed well in immunohistochemical analysis. Nevertheless, we tried the best antibody from our screen on a prostate cancer tissue array but did not obtain any usable results. Although we currently lack immunohistochemical data, we believe the anti-correlation of ERG and VCL mRNA levels in 5 clinical prostate cancer datasets (including the extensive MSKCC dataset) should serve as an adequate preliminary (although not fully compelling) evidence for the anti-correlated relationship between ERG and VCL expression in prostate cancers.

Panel J: These data, which have the potential to provide biological relevance, are underdeveloped. Images should be shown to support the data shown, and alternative model systems should be used to assess the impact on cell migration/invasion.

As requested, we have now included the images to support our data (Fig. S10). We have also knocked-down VCL in LNCaP cells and observed a similar increase in the invasive capability of VCL in prostate cancer cells (Fig. S11).

Referee #3 (Remarks to the Author):

Major points:

1. *The notions summarized above go against the grain because of the generally accepted insight that INCREASED AR activity (such as AR amplification and activity as measured by PSA expression) is associated with prostate cancer progression, even including ablation resistant disease. These issues need to be explicitly and specifically addressed in terms of prostate tumours (and not only in cell lines).*

We understand the reviewer's concerns. Indeed, the activity of AR is of paramount importance to prostate cancer progression. Multiple studies have demonstrated the role of AR in promoting survival and proliferation in prostate cancer cells (Schiewer et al., Mol Cell Endocrinol. 2011; Liao et al., Mol Cancer Ther. 2005). Moreover, a functional and active AR has widely been shown to be crucial for the development, growth and survival of hormone refractory prostate cancers (Wang et al., Cell 2009; Chen et al., Nat Med 2004). Through acquiring AR amplification, prostate cancer cells could also become more sensitive to androgens and thus could respond to basal levels of androgens for proliferation and survival (Chen et al., Nat Med 2004). We too are in agreement with the generally accepted insights. In fact, a recent study from our lab demonstrated the importance of AR activity in promoting prostate cancer cell survival (Tan et al., Mol Cell Biol. 2012). However, we think the results from our current study do not go against these general notions but instead provide an extension of the above concepts.

Our current data argue that while an enhanced level of AR activity is required for cell survival and proliferation, hyperactivation of AR might not be beneficial for prostate cancer progression to a metastatic form. Accordingly, androgen signaling has to be modulated to an optimal level in prostate cancer cells for proliferation, survival and metastasis. In this study (Fig. 7A), using Molecular Concept Map Analysis, we demonstrated that "a defined set" of ERG-associated androgen upregulated genes was significantly associated with gene signatures that are overexpressed in prostate primary tumors (compared to normal prostate) but repressed in metastatic and high-grade prostate cancers (compared to primary prostate tumors). This result implies a reduced androgen signaling (within a restricted set of androgen-regulated genes) in aggressive forms of prostate cancers in comparison to primary prostate tumors. In fact, this work corroborates the findings of a recent study by another group using a similar approach but with their own defined AR target gene signature (Yu et al., Cancer Cell 2010). Interestingly, the androgen receptor was also recently shown to suppress its own expression when stimulated by high levels of androgens (Cai et al., Cancer Cell 2011). This possibly suggest that hyperactivation of AR might actually not be favorable for prostate cancer progression.

To address the reviewer's concern in greater detail and to further support the clinical significance of our work, we have now included an additional analysis of an extensive clinical prostate cancer dataset recently published by *Taylor et al., Cancer Cell 2010*. We show that the same set of ERG-associated androgen upregulated genes defined in our original manuscript displayed significantly higher expression in primary prostate tumors (compared to normal prostate) but significantly lower expression in metastatic prostate tumors (compared to primary tumors) (Fig. S7). Although further detailed investigations are required, we believe we have provided significant preliminary clinical evidence to support our claim.

2. *The manuscript is littered with many imprecise and sloppy statements.*

We have made numerous changes throughout the manuscript to correct for any inaccuracies.

Minor points:

1. *Paragraph 1 of the introduction relies on two old references (Heinlein & Chang, 2002 & 2004). Better (and more recent) references exist.*

We have now replaced these references with more recent references (Shen et al., Genes and Dev. 2010, and Schiewer et al., Mol Cell Endocrinol. 2011).

2. *The colour differences in Fig 1C are not distinct enough and can be confusing.*

We have made changes to the color scheme to make the graphs stand out more.

3. *In Fig. 1 the expression analyses of the AR and ERG were at 6, 12 and 24 hours after DHT treatment, yet the times for the ChIP-seq analyses were done after 2 and 18 hours after DHT treatment. To interpret the latter the expression levels of the two transcription factors are needed at the same times, especially as expression changes quite significantly over time.*

Fig. 1A was only to show ERG is an androgen gene as reported previously by others. The main purpose of Fig. 1 was to show that from our western analysis of VCaP cells there is a high level of ERG present in VCaP cells 0-2 hrs after DHT stimulation and this level is significantly further increased after 18 hrs. It was based on this western result that we decided to perform our ChIP-Seq at 0, 2 and 18 hrs. Thus, the more important comparison is the protein levels of when the ChIP-Seq was performed as shown in Fig. 2B.

4. *It is stated near the top of page 7: "Finally, we noticed that AR recruitment was significantly stronger at AR+ERG co-localized binding sites compared to AR unique sites (Fig. 2G)". What exactly is meant by "stronger"? Higher affinity or more sites occupied in the mixture of cells? Binding is typified by being "stronger" or "weaker" at many places throughout the manuscript.*

We apologize for the confusion. Stronger binding is typified as exhibiting higher tag counts (for ChIP-Seq) and higher % input (for ChIP-qPCR). We have now included this additional information in the main text.

5. *Towards the bottom of page 7 it is stated: "...our AR ChIP-seq showed ARBS are preferentially located at distal regions that are far away from the transcriptional start sites (TSS) of genes (Fig.2E)". The % of binding sites at the different genomic regions needs to be compared with random regions of about the same size to be able to make the above statement (especially typifying the binding as "preferentially located").*

We agree. We have now included random regions in our analysis and found minimal difference between the random regions and ARBS (Fig. 2G). Accordingly, we have now changed our conclusion to “our AR ChIP-Seq showed ARBS are mostly located at distal regions that are far away from the transcriptional start sites (TSS) of genes”. We thank the reviewer for bringing this to our attention.

6. *Further down on page 7 it is stated: "In conservation analysis, AR and ERG are generally more conserved at the ChIP-Seq peak center relative to their flanking regions (Fig. 2F)." What else was expected?*

We apologize for the confusion. We were analyzing whether there was any evolutionary conservation at the DNA level for the different groups of AR and ERG binding sites. For this, we used the flanking regions of the binding sites as background control. Thus, if the ChIP-Seq peak center is of a higher conservation score than its surrounding regions, we considered the binding sites to be generally and relatively more conserved through evolution and hence might be functionally important.

7. *In the section "Transcriptional collaboration between AR and ERG", only two sites/genes were analyzed (PSA and FKBP5). More sites/genes need to be considered before the collaboration can be generalized.*

We agree. Please refer to reviewer 1, comment #4.

8. *Bottom of page 10 it is stated: "In contrast, ERG unique binding sites preferred to recruit HDAC1 and -2, but not EZH2." Personification of inanimate objects needs to be avoided and statements like these (also elsewhere) need to be rephrased.*

We have made the changes accordingly.

2nd Editorial Decision 28 March 2012

Thank you very much for the revised study. Two of the original referees assessed the rather significant revisions and are fully satisfied with the improvements provided.

The editorial office will soon be in touch with necessary paperwork related to official acceptance.

Please allow me to congratulate to the study.

Yours sincerely,

Editor
The EMBO Journal